

Somatic *TP53* Mutations Are Relatively Rare among Adrenocortical Cancers with the Frequent 17p13 Loss of Heterozygosity

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Abstract Purpose: Allelic losses [loss of heterozygosity (LOH)] at the 17p13 locus are frequent (85%) in adrenocortical cancers. The tumor suppressor gene *TP53* is located at 17p13. The aim of the study was to determine the frequency of *TP53* somatic inactivating mutations in adrenocortical tumors with 17p13 LOH and their clinico-biological correlations.

Experimental Design: *TP53* somatic mutations, intragenic LOH (VNTR1 marker), and p53 overexpression were studied in 36 adrenocortical tumors with 17p13 LOH determined by Southern blot.

Results: *TP53* mutations were detected in 33% of the tumors, and VNTR1 LOH was present in 44% of the cases and did not always correlate with the presence of a *TP53* mutation. Only the *TP53*-mutant tumors exhibit a strong nuclear immunoreactivity. *TP53*-mutant tumors were significantly larger than wild-type *TP53* tumors (median tumor weight: 640 versus 185 g; $P = 0.02$), were associated with a more advanced stage of tumor progression (MacFarlane stage IV; $P = 0.01$), and had a shorter disease-free survival ($P = 0.03$).

Conclusions: The finding that only a minority of adrenocortical tumors with 17p13 LOH had either a VNTR1 LOH or a *TP53* mutation indicates that *TP53* might not be the only or major tumor suppressor gene at 17p13 involved in adrenocortical cancer progression. We suggest that a genetic instability of the 17p13 region, occurring early in adrenocortical cancer development, involves various genes located in this region. *TP53* might be only one of them, and its alteration by the occurrence of inactivating mutation is associated with the development of more aggressive tumors.

Adrenocortical cancer is a rare disease with a poor prognosis. The 5-year survival rate varies between 16% and 38% (1, 2) among series. Adrenocortical cancers are frequently considered as heterogeneous tumors with various clinical presentations

and outcome. This heterogeneity could reflect different mechanisms of tumor development. The pathogenesis of adrenocortical cancer is still poorly understood, although significant progress has recently been made (3). Adrenocortical cancers are monoclonal tumors containing multiple chromosomal alterations (see refs. 3, 4 for review). The study of genetic syndromes predisposing to adrenocortical tumors has been of great help to identify the molecular alterations that are also present in sporadic tumors (5–8). Loss of heterozygosity (LOH) at 17p13 is among the chromosomal alterations described in adrenocortical cancers. 17p13 LOH is observed in 85% of adrenocortical cancer and almost never in adenomas and therefore could be used as a molecular marker of malignancy, along with 11p15 alterations and insulin-like growth factor-II overexpression (6).

The occurrence of an allelic loss suggests the presence of a tumor suppressor gene that would be inactivated by this chromosomal alteration. *TP53* is a tumor suppressor gene located at 17p13.1 and is involved in the control of cell proliferation. Germ-line mutations of *TP53* are identified in 70% of families with the Li-Fraumeni syndrome, characterized by breast carcinoma, soft tissue sarcoma, brain tumors, osteosarcoma, leukemia, and adrenocortical cancer (9). *TP53* germ-line mutations have been observed in 50% to 80% of children with apparently sporadic adrenocortical cancer in North America and Europe (10, 11). The incidence of pediatric adrenocortical cancer is ~ 10 times higher in southern Brazil than in the rest of

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the world, and a specific germ-line mutation has been identified in exon 10 of the *TP53* gene (R337H; refs. 12, 13). In sporadic adrenocortical cancer in adults, somatic mutations of *TP53* are found in only 25% of adrenocortical cancer cases and are located in four "hotspot regions" within exons 5 and 8 as first shown by Ohgaki et al. (14) and Reincke et al. (5) in small series and more recently by Sidhu et al. (15). A recent report from Italy describes a *TP53* mutation rate of 70% in a series of 10 adrenocortical cancers (16). *TP53* mutations are believed to be involved in tumorigenesis or tumor progression and have been reported to be associated with aggressiveness or poor prognosis of lung, ovarian, and colon tumors (17–19). Although the study of p53 by immunohistochemistry is not a highly reliable indicator of the presence of mutations, it has been associated with poor prognosis (20, 21). However, the role and importance of somatic mutation of *TP53* in sporadic adrenocortical cancer of adults has not been completely investigated. Furthermore, despite the location of *TP53* at 17p13, it is not established that it is indeed the tumor suppressor gene involved in these frequent chromosomal losses in adrenocortical cancer.

The aim of this study was to determine, in a large cohort of adult sporadic adrenocortical tumors selected for 17p13 LOH, the rate of *TP53* mutations and to precise the phenotype associated with these somatic mutations. We found that one third of such adrenocortical tumors harbor a *TP53* mutation that is associated with a more aggressive and advanced tumor.

Materials and Methods

Patients. Thirty-six patients [29 women and 7 men; age (mean \pm SD), 44.7 \pm 16.2 years] with adrenocortical tumors presenting a 17p13 LOH determined by Southern blot as previously reported (6) were included in this study. The patients were followed until the date of their death, their last examination, or the end of the follow-up period (1–119 months). Twenty-five of 36 (69%) were glucocorticoid-secreting tumors and 15 of 25 (60%) were also androgen-secreting tumors. Five of 36 (14%) were only androgen-secreting tumors, and 1 was a glucocorticoid- and estrogen-secreting tumors. Six of 36 (17%) were incidentally discovered adrenal masses.

The stage of the tumor was assessed according to the MacFarlane classification (22). Pathologic data were assessed according to Weiss criteria: for each patient, a Weiss score (0–9) was determined according to nine histologic features (23). Informed signed consent for the analysis of leukocyte and tumor DNA and for access to the data collected was obtained from all the patients, and the study was approved by an Institutional Review Board (Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale, Cochin Hospital, Paris, France).

Tumors. Tumor fragments obtained during surgery were immediately frozen and stored in liquid nitrogen until DNA extraction. For diagnosis and scoring, tumors were fixed in formalin and embedded in paraffin, and 4- μ m sections were cut and stained with H&E.

DNA and RNA preparation. Nucleic acids (DNA and RNA) were prepared from surgically removed adrenocortical tumors as described previously (24). The High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA) was used for reverse transcription-PCR.

PCR amplification of tumoral DNA and cDNA. The 10 coding exons (exons 2–11) and the flanking intronic sequences of the *TP53* gene (Genbank accession no. U94788) were amplified by PCR using the following specific primers: exons 2 to 3, 5'-CAGGGTTGGAAGTGTCTC-3' (sense) and 5'-GCACTGTAGATGGGTGAA-3' (antisense); exon 4, 5'-CGTTCTGGTAAGGACAAG-3' (sense) and 5'-CTAAGGGTGAAGAGGAAT-3' (antisense); exon 5, 5'-GTTTGTTCITTTGCTGC-3' (sense) and

5'-CAGACCTAAGAGCAATCA-3' (antisense); exon 6, 5'-CAGATAGC-GATGGTGAAG-3' (sense) and 5'-CACTGACAACCACCCTTA-3' (antisense); exon 7, 5'-TGCCACAGGTCTCCCAAGG-3' (sense) and 5'-GGTAGTAGTATGGAAGAAAT-3' (antisense); exon 8, 5'-GGTTGGG-AGTAGATGGAG-3' (sense) and 5'-TGAATCTGAGGCATAACT-3' (antisense); exon 9, 5'-CTAAGCGAGGTAAGCAAG-3' (sense) and 5'-GAGCCATTGTCTTTGAGG-3' (antisense); exon 10, 5'-CCATCTTT-TAACTCAGGT-3' (sense) and 5'-GAAGCAGGATGAGAATG-3' (antisense); and exon 11, 5'-TCCCCTGTGCCAGCCTTAG-3' (sense) and 5'-CAAGCAAGGGTCAAAGA-3' (antisense). The PCR conditions were as follows: after denaturation at 94°C for 5 min, PCR consisted of 35 cycles of 30 s at 94°C, 30 s at 54°C to 56°C, and 30 s at 72°C and subsequently followed by a final extension step of 5 min at 72°C. All amplified samples were examined by agarose gel electrophoresis to confirm successful amplification of each exon of *TP53*. Direct sequencing of the purified fragments was then done using the Genetic Sequencer ABI3100 Applied Biosystems apparatus. The mRNA of four *TP53* mutated tumors without LOH at VNTR1 (nos. 5, 6, 10, and 12) was amplified by PCR using appropriate specific primers. Subsequently, the purified fragments were directly sequenced to identify the *TP53* alleles expressed.

LOH analysis of VNTR1 marker. To assess the LOH in the adrenal tumors, a *TP53* intragenic highly polymorphic marker, VNTR1 (pentanucleotide repeat, 118–130 bp in size), was used (25). The VNTR1 microsatellite is located at 7,522,600 bp from the centromere (7.5 Mb). It is located between the 17S960 and 17S1353 microsatellite and is adjacent to the dinucleotide repeat polymorphism p53CA. The VNTR1 was chosen because of its high informativity and location within *TP53* gene itself. Briefly, leukocyte and tumor DNA were amplified by PCR with the use of fluorescent-labeled primers. Analysis of the PCR product was done with an automatic sequencer (model CEQ 8800, Genetics Analysis System version 8.0; Beckman Coulter, Fullerton, CA).

Immunohistochemistry. Section of 4 μ m from formalin-fixed tissue embedded in paraffin was mounted on Superfrost/Plus glass slides. The paraffin was eliminated by incubating the sections in xylene and then rehydrating them. For antigen retrieval, sections were heated in a microwave oven for a total of 20 min in 10 nmol sodium citrate buffer at pH 6.0. The slides were incubated with monoclonal anti-p53 antibody (DO-7, DAKO, Glostrup, Denmark) at a dilution of 1:400 for 60 min at room temperature. Sections were then incubated with the streptavidin-biotin-peroxidase complex, and the marker was detected by the enzymatic precipitation of the 3,3'-diaminobenzidine tetrahydrochloride in 0.5 mmol Tris. The slides were counterstained with Mayer's hematoxylin. Immunostaining was assessed blinded to MacFarlane stage, Weiss score, *TP53* mutation, and outcome. The p53-stained sections were all examined at high magnification, and a labeling index (percentage of stained cells) was attributed to each case. The intensity of staining was not scored.

Statistical analysis. Relationships between *TP53* status and categorical variables were tested using the χ^2 test or the Fisher's exact test when appropriate. Relationships between *TP53* status and continuous variables were tested using the Student's *t* test or the Wilcoxon's signed rank test when appropriate.

Overall survival was calculated from the date of surgery to death or last follow-up. Disease-free survival was defined as the time elapsed from the date of surgery to the first relapse or death or last follow-up. Patients were censored if they had not experienced the end point of interest at the time of last follow-up. Survival curves were derived from Kaplan-Meier estimates. Log-rank test was used to compare survival distributions between subgroups. Cox proportional hazards regression model was done to estimate the prognosis effect of continuous variables in univariate analysis and to adjust the prognostic value of *TP53* status for biological and clinical features.

All statistical analyses were done using R software package. Statistical significance was considered as *P* values of <0.05, and all tests were two sided.

Table 1. Summary of TP53 mutations and VNTR1 LOH in the 12 adrenocortical tumors with TP53 mutation

Tumor no.	LOH VNTR1	Exon, n	Codon base	Consequences of the mutation
1	Yes	5	12 bp del 129*	4 amino acid deletion
2	Yes	5	1 bp del 153	Frameshift after codon 153; stop codon after 16 missense residues
3	No	5	152, CCG→CTG*; Pro→Leu	Missense
4	No	5	154, GGC→GTC*; Gly→Val	Missense
5	No	5	132, AAG→AAC*; Lys→Asn	Missense
6	No	5	176, TGC→TTC*; Cys→Phe	Missense
7	No	4	60, CCA→ACA*; Pro→Thr	Missense
		8	274, GTT→TTT*; Val→Phe	Missense
8	Yes	4	7 bp ins 53	Frameshift after codon 53; stop codon at codon 53
9	Yes	6	3 bp del 213; 2 bp ins 213	Frameshift after codon 213; stop codon after 33 missense residues
10	No	8	283, CGC→TGC*; Arg→Cys	Missense
11	Yes	10	358, GAG→GTG*; Glu→Val	Missense
12	No	10	346, GAG→TAG*; Glu→Stop	Nonsense

*Denotes mutation described in the IARC TP53 database (<http://www-p53.iarc.fr>).

Results

TP53 mutations and LOH. A TP53 mutation was found in 12 of 36 (33%) tumors. Two mutations were observed in tumor no. 7: one in exon 4 and one in exon 8. Exon 5 was the most often involved (six tumors) followed by exon 4, 8, and 10 (each mutated in two tumors) and exon 6 (one tumor; Table 1; Fig. 1A).

Nine of 13 were point mutations: 8 (tumors nos. 3, 4, 5, 6, 7, 10, and 11) were missense mutations and 1 (tumor no. 12) was a nonsense mutation. Three of 13 mutations (tumor nos. 2, 8, and 9) were a deletion or an insertion of a single/multiple nucleotide leading to a frameshift and a premature stop codon; one was a deletion of 12 bases leading to a deletion of four amino acids without the appearance of a premature stop codon.

Twelve (33%) other tumors presented a TP53 polymorphism: 9 at exon 4 (Arg⁷²Pro in 8 cases and Pro³⁶Pro in 1 case) and 3 at exon 6 (Arg²¹³Arg).

Allelic losses on the TP53 locus, as determined using the intragenic VNTR1 microsatellite marker, were observed in 16 of 35 (45%) informative cases: 5 had TP53 mutation and 11 had no TP53 mutation; therefore, the frequency of VNTR1 LOH did not correlate with the presence of a TP53 mutation (Table 2; Fig. 1A). An example of VNTR1 LOH is shown in Fig. 1B.

To detect the allelic status in the TP53 mutated tumors without VNTR1 LOH, the reverse transcription-PCR product of four tumors (nos. 5, 6, 10, and 12) was sequenced. In three of four cases, only the mutant allele was present, whereas both mutant and wild-type allele were detected in one case. Notably, in all these cases, a p53 nuclear overexpression was present (Fig. 1A).

p53 expression. Immunohistochemistry was used to examine the cellular localization of p53 in 16 adrenocortical tumors: 6 with a TP53 somatic mutation, 5 with a TP53 polymorphism, and 5 without TP53 mutation. Interestingly, a nuclear staining was present only in all the tumors with a TP53 mutation, with a

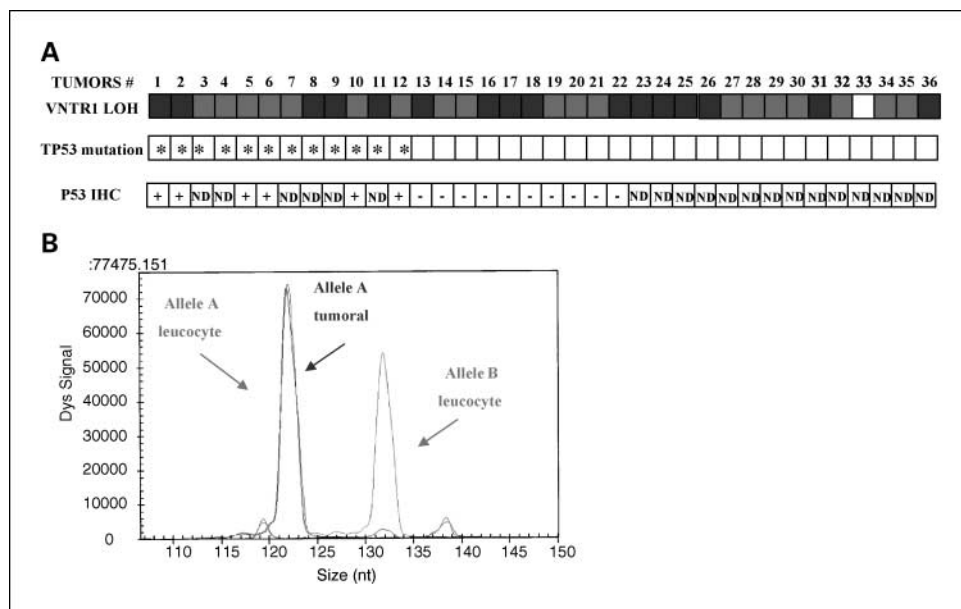


Fig. 1. Results of VNTR1 LOH, TP53 mutations, and p53 immunohistochemistry. **A**, overall molecular characteristics of the 36 adrenocortical tumors: VNTR1 allelic status: LOH (■), allelic retention (▣), not informative (□), TP53 mutations (*), and p53 overexpression (+). IHC, immunohistochemistry; ND, not done. **B**, the microsatellite marker is informative because two different alleles (alleles A and B) are observed at the level of the leucocyte (germ line) DNA. By contrast, analysis of the tumor DNA shows only one allele (allele B), showing that LOH occurs at this locus in this adrenocortical cancer.

Table 2. Clinical, immunohistochemical, and molecular variables between tumors with and without TP53 mutations

	Tumors with TP53 mutation	Tumors with wild-type TP53	P
Patients, n (%)	12 (33)	24 (66)	—
Age, y (mean \pm SD)	46 \pm 16.5	44.1 \pm 16.3	NS*
Glucocorticoid secretion, n (%)	10 (83)	15 (63)	NS
Tumor weight, g [median (min-max)]	640 (281-2,200)	185 (43-2,700)	0.02
Weiss score \geq 4, n (%)	10 (83)	19 (83)	NS
MacFarlane score 2 vs 3-4, n (%)	9 (75)	8 (33)	0.02
VNTR1 LOH, n (%)	5 (42)	11 (46)	NS
11p15 LOH, n (%)	9/11 informative cases (81)	21/23 informative cases (91)	NS
IGF-II overexpression, n (%)	9/12 (75)	18/22 (82)	NS
p53 overexpression by immunohistochemistry, n (%)	6/6 (100)	0/10 (0)	<0.01

Abbreviation: IGF-II, insulin-like growth factor-II.

*Nonsignificant ($P > 0.05$).

variable percentage of positive cells ranging from 5% to 60%. In the tumors with a TP53 polymorphism or without TP53 mutation, no staining for p53 was detected (<1% of cells; Fig. 2).

Correlation between TP53 mutations and clinical variables. Clinical features were compared between the patients bearing mutated or nonmutated tumors (Table 2). There was no statistical difference in age of the patients between the tumors with and without TP53 mutation (46 \pm 16.5 versus 44.1 \pm 16.3 years).

TP53-mutant tumors were larger [median tumoral weight: 640 g (minimum-maximum, 281-2,200) versus 185 g (minimum-maximum, 43-2,700)]; $P = 0.02$] and had a more advanced MacFarlane stage of tumor progression. Therefore, metastases (MacFarlane stage IV) were more frequent in patient presenting a TP53-mutant tumor (stage IV: 67% versus 21%; $P = 0.01$). No significant difference in Weiss score was observed. There was a nonsignificant higher percentage of glucocorticoid-secreting tumors in the TP53-mutant group (10 of 12, 83%) versus the TP53 wild-type group (15 of 24,

63%). In both TP53-mutant and TP53 wild-type tumors, a high percentage of 11p15 LOH and insulin-like growth factor-II overexpression were shown as expected from malignant adrenocortical tumors (6) without significant difference between the two groups.

Survival analysis. During a median duration of follow-up of 63 months (minimum-maximum, 1-119 months), 20 of 36 patients died. Eleven had metastasis at diagnosis (MacFarlane stage IV) and 9 presented with tumor recurrence 3 to 24 months after surgery. Among the 16 patients alive, 2 had metastases (MacFarlane stage IV) at diagnosis and 4 displayed recurrence 10 to 24 months after surgery.

Tumors with a TP53 mutation had a shorter disease-free survival as determined by univariate analysis ($P = 0.03$; Fig. 3A). Nevertheless, overall survival was not significantly different between the TP53-mutant and TP53 wild-type tumors ($P = 0.25$, log-rank test). High MacFarlane stage ($P = 0.001$) and high tumor weight ($P = 0.001$) were associated with a shorter overall survival (Fig. 3B and C).

Discussion

Allelic losses at 17p13 are frequent in various type of tumors, such as breast (26), colon (27), and hepatocellular cancers (28), with a variable prevalence ranging from 40% to 60%. Interestingly, our group has shown that 17p13 LOH is even more frequent in adrenocortical cancer and could be detected in 85% of these adrenal neoplasms (6). Because 17p13 LOH is rare in benign adrenocortical tumors and that pathologic diagnosis of malignancy can be difficult in adrenal neoplasms, we have suggested the use of 17p13 LOH analysis as a molecular marker of malignancy. The tumor suppressor gene TP53 that maps in this region is clearly implicated in the tumorigenesis of several types of cancers, and loss of p53 function could result from mutational events, allelic losses, and epigenetic alterations.

To assess further the exact role of p53, we have studied TP53 mutations, p53 expression, LOH within the TP53 gene, and the correlation with clinical characteristics in a large cohort of tumors displaying 17p13 LOH.

In previous studies done in sporadic adrenocortical cancer, the TP53 mutation rate is usually rather low (25%), except in a single series (70%; refs. 3, 5, 14, 16). However, most of these

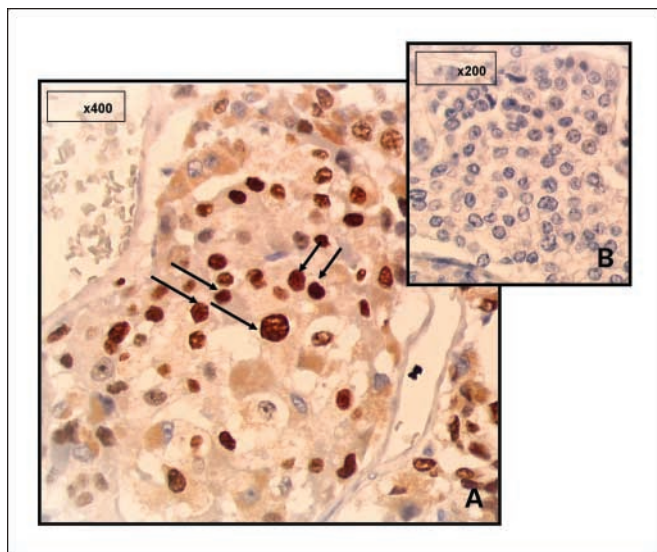


Fig. 2. p53 immunohistochemistry. p53 overexpression in a TP53-mutant tumor showing strong nuclear staining. A, arrow, dark-brown nuclear staining. Magnification, $\times 400$. No p53 overexpression in a TP53 wild-type tumor. B, blue negative nuclear staining. Magnification, $\times 200$.

studies were done in small series of 11 to 15 adrenocortical tumors and only exons 5 to 8 were sequenced, except in the work by Sidhu et al. (15). Here, by sequencing all the 10 coding exons of a larger group of adrenocortical tumors, we showed that *TP53* somatic mutations are present in a third of the tumors. As shown for other type of tumors (17, 29, 30), most of the *TP53* mutations are within exons 5 to 8 (11 of 13 mutations), corresponding to the core-binding domain of the p53 protein. A previous study showed an important percentage of mutations located in exon 4 in adrenal adenomas of Taiwanese patients (31), which was not confirmed in another study of adrenocortical tumors from Europe (32). On the contrary, in our series of adrenocortical cancer, we found two different mutations at exon 4 probably due to larger size of the cohort of tumors studied than in the former. The exon 10 (R337H) mutation is very frequently found on germ-line DNA in children with adrenocortical cancer in southern Brazil (12, 13, 33) but was not detected at the somatic level in any patient of this cohort of adult sporadic adrenocortical tumors from a European country. In previous studies, the codon 72 polymorphic variants in the exon 4 of *TP53* have been shown to have a markedly different apoptotic potential (34–37). However, in this study, the tumors harboring this codon 72 variant do not seem more aggressive (data not shown).

In this study, the LOH at the *TP53* locus is determined using the VNTR1, a highly polymorphic marker present in the *TP53* promoter. A LOH within the *p53* gene was found in only 44% of the tumors, whereas all had 17p13 LOH. Furthermore, in only 5 of the 12 *TP53* mutated tumors, a VNTR1 allelic loss was detected. Such discrepancy has been described in hepatocellular carcinomas (28) and gastric cancers (38). The presence of VNTR1 LOH without *TP53* mutations could suggest that the tumor is still in an early stage. On the other hand, some tumors may display a *TP53* mutation without LOH at 17p13 as observed in breast cancer (28, 39, 40), hepatocellular carcinoma (28), oral squamous cell carcinoma (30), and gastric cancer (38). In our series, the study of mRNA in four of seven mutated tumors without VNTR1 LOH showed that, in three of four, only the mutated allele is present.

The lack of LOH at the *TP53* locus might imply other mechanisms of *TP53* wild-type allele inactivation, such as methylation alteration. *TP53* methylation alterations might be implicated in acute lymphoblastic leukemia (41), in brain metastasis of solid tumors (42), and in gliomas (43). However, no alteration was found in hepatocellular carcinoma (44) and neuroblastic tumors (45). In adrenocortical cancers, *TP53* promoter methylation alterations have not been found by Sidhu et al. (15). To explain the lack of LOH, a speculative explanation might also be a dominant-negative effect of the mutant *TP53* protein over the wild-type (46).

Interestingly, in our series, the *TP53* mutations are always associated with p53 accumulation as determined by immunohistochemistry. This agrees with previous observations in other tumors, such as gastric and breast cancers (21, 47), although this finding is not constant in cancers (17, 20, 48, 49). Until now, few data were available on adrenocortical tumors, and the correlation between p53 overexpression and *TP53* mutations was unclear probably due to the small number of studied tumors (5, 16). The correlation between p53 accumulation and the clinical behavior and survival of patients is clearer as shown

by Sredni et al. (50). Notably, in our series, a recurrence occurred between 7 and 15 months after initial surgery with complete tumor removal in four of six patients carrying a tumor with p53 overexpression.

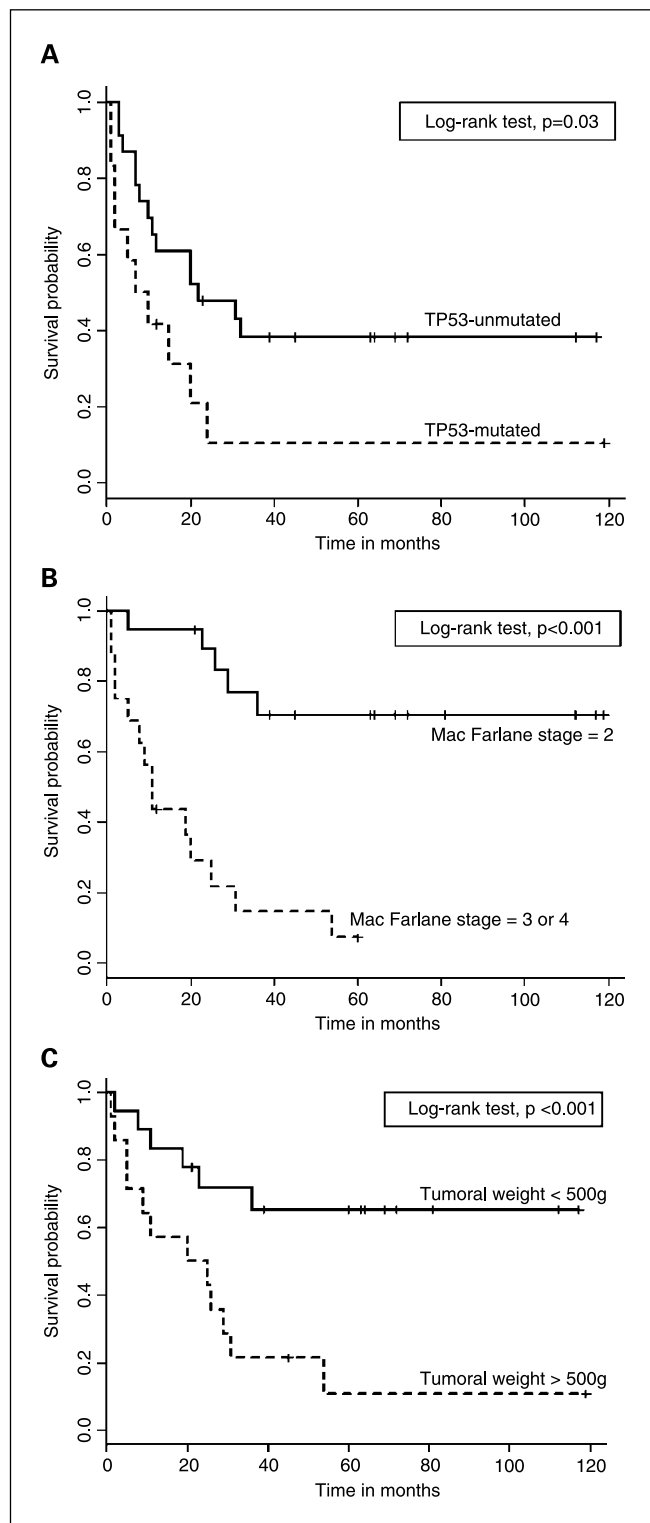


Fig. 3. Kaplan-Meier analysis of disease-free survival and survival. *A*, Kaplan-Meier disease-free survival curves after initial surgery according to the presence of *TP53* mutation. $P = 0.03$, log-rank test. *B* and *C*, Kaplan-Meier overall survival curves according to the MacFarlane stage (*B*) and tumoral weight (*C*). $P < 0.001$, log-rank test.

The analysis of the clinical and histologic variables allowed to define some important differences between the *TP53* mutated and wild-type tumors. The MacFarlane score and the tumoral weight are significantly higher in *TP53* mutated tumor, whereas no significant difference in the patient age or secretion pattern is found. Furthermore, *TP53* mutations are associated with a shorter disease-free survival.

The high prevalence of 17p13 LOH in malignant adrenocortical tumors suggests that this is an early event in malignant progression. The observation that a minority of adrenocortical tumors with 17p13 LOH exhibit allelic losses within the *TP53* gene, as determined using the VNTR1 marker, suggests that *TP53* might not be the only or major tumor suppressor gene involved in early adrenocortical tumor progression. This is in keeping with the low frequency of *TP53* somatic mutations in adrenocortical tumors with 17p13 LOH because they are present in only a third of these tumors. However, these *TP53* mutations are found in larger and advanced tumors. This

suggests that *TP53* mutations are a late event associated with a more aggressive phenotype in adrenocortical tumor progression. This would imply a model with a genetic instability of the 17p13 region occurring early in adrenocortical tumors involving various genes located in this region. *TP53* might be one of them and its alteration by the occurrence of inactivating mutation might be a late event associated with a more aggressive phenotype. The identification of the other tumor suppressor genes involved in 17p13 promises to be important for the progress in the pathophysiology of adrenocortical tumors as previously suggested for other types of cancers (26–28, 39).

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